Phenylbutyrate Induces Apoptosis in Human Prostate Cancer and Is More Potent Than Phenylacetate

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ABSTRACT
Phenylbutyrate (PB), a novel lead compound for prostate cancer therapy, has molecular activities distinct from its metabolite, phenylacetate (PA). Both PB and PA promote differentiation in human prostate cancer cell lines, yet little data exist comparing the cytotoxic effects of each drug. We found that PB is more potent than PA in vitro; PB is 1.5–2.5 times more active at inhibiting growth and inducing programmed cell death than PA at clinically achievable doses against each human prostate cancer line studied. PB is equipotent to sodium butyrate, which induces apoptosis and differentiation through multiple mechanisms. Exposure of prostate cancer cell lines to PB reduces their DNA synthesis, leads to fragmentation of genomic DNA, and causes 50–60% of cells to undergo apoptosis. These PB-induced effects are 2–10 times greater than those of the control or PA. The stereotypical changes of apoptosis can be seen with sodium butyrate at similar concentrations, but not with PA. Prostate cancer cell lines overexpressing P-glycoprotein or possessing heterogeneous molecular alterations, including p53 mutations, are also sensitive to the effects of PB. In vivo, Copenhagen rats treated with oral PB had delayed growth of the androgen refractory Dunning R-3327 MAT-LyLu prostate cancer subline by 30–45% in a dose-dependent manner. These results demonstrate that PB induces cytotoxicity via apoptosis in human prostate cancer, in addition to its differentiating properties.

INTRODUCTION
The phenyl fatty acids PA and PB are potent differentiating agents (1). Samid and colleagues (2–6) have demonstrated differentiation and growth inhibition induced by PA or PB in malignant glioma, prostate cancer, leukemia, melanoma, and neuroblastoma cell lines in vitro and in vivo. These differentiating agents also induce fetal hemoglobin production in children with sickle cell anemia and thalassemia (7, 8), are used in children with urea cycle disorders (9, 10), and seem to be nontoxic. In addition, these drugs have been used in adults with idiopathic hyperammonemia syndrome after high dose chemotherapy without significant end organ toxicities (11).

PB is β oxidized in vivo to PA in mitochondria of the liver and kidney rapidly and provides a more tolerable delivery of PA (12–14). PB seems to have cellular and molecular effects distinct from PA. In vitro studies provide insight to the unique effects of each agent, because PB is not β oxidized in vitro to PA in prostate cancer cell lines. Wood et al. (15) reported that PA increases, whereas PB decreases, PSA protein production by the LNCaP prostate cancer cell line (15). Theoretically, differentiation could increase PSA secretion, whereas cytotoxicity leads to a decline in PSA production. On the basis of these data, we tested the hypothesis that PB, unlike PA, may promote apoptosis as well as differentiation. In this respect, PB may be analogous to BU, a short chain fatty acid, which is a differentiating agent and which also demonstrates cytotoxic effects in vitro and in vivo (16–18).

To investigate this hypothesis, PA, PB, and BU were tested for cytotoxicity against human prostate cancer cell lines, including those with acquired p53 mutations and those selected for MDR1 overexpression. The potency of each agent was tested in the range of clinically achievable levels (1–5 μM) in humans. The ability of these agents to induce apoptosis in human prostate cancer was assayed in vitro directly. Dose-dependent cytotoxic effects were studied in vivo in the Dunning rat MAT-LyLu prostate cancer model.

MATERIALS AND METHODS
Growth Inhibition and Cytotoxicity Assays Comparing Potency of PB, PA, and BU against Human Prostate Cancer Cell Lines

The human prostate cancer cell lines LNCaP-ATCC, LNCaP-GW, PC-3, PPC-1, and TSU-Adr 1000 (obtained from W. G. Nelson) and the Dunning rat R-3327 MAT-LyLu subline were plated uniformly in 96-well plates (5 × 10^3 cells/well). PB and PA (Triple Crown America, Perkasie, PA) and BU (Sigma Chemical Co., St. Louis, MO) were formulated in RPMI 1640 (GIBCO-BRL, Gaithersburg, MD) with 10% FCS (IRH Biochemicals, Lenexa, KS). After 96 h of drug exposure, treated cells were fixed and stained with 5% methanol and 0.5% crystal violet, air dried, and resolubilized with 1% SDS. Cell density was assayed by spectrophotometer at 590 nm (Bio-Rad 450 Micro plate reader; Bio-Rad, Hercules, CA). Each condition was expressed as

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3 The abbreviations used are: PA; sodium phenylacetate; PB, sodium phenylbutyrate; BU, sodium butyrate; PSA, prostate-specific antigen; MDR, multidrug resistance; IC_{50}, 50% inhibitory concentration.

4 H. Shim and J. Wehrle, personal communication.
Fig. 1  Composite inhibitory potency of PB, PA, and BU against prostate cancer cell lines. Growth inhibition and cytotoxicity as proportionate survival (i.e., 0.4 = 40% inhibition) are plotted against varying concentrations of each agent. Inhibition after treatment with PA, BU, and PB is shown. Cell lines: A, MLL-MAT-LyLu (MLL) rat prostate cancer; B, TSU human prostate cancer; C, PC-3 human prostate cancer; D, PPC-1 human prostate cancer; E, LNCaP-ATCC human prostate cancer; and F, LNCaP-GW (p53 mutant) human prostate cancer.
an average of eight determinations for that concentration of the drug.

Phenotypic Changes of Human Prostate Cancer Cell Lines after Exposure to PB, PA, and BU

Each prostate cancer cell line was plated on eight-chamber glass slides (Nunc, Inc., Naperville, IL), allowed to adhere, and exposed to 2.5 and 5 mM concentrations of the drugs. After 72 h of exposure, cells were fixed in 100% ethanol and stained with hematoxylin and eosin.

Quantitation of DNA Synthesis after PB Treatment

The androgen-independent human prostate cancer cell line TSU was exposed to 2.5 mM PB for varying time intervals from 30 min to 96 h. After each time interval, cells were labeled with 1 μCi/ml [3H]thymidine (Amersham, Arlington Heights, IL). Prostate cancer-precipitable radioactivity was determined using a liquid scintillation counter. The concentration of DNA for each sample was determined using diphenylamine as described previously (19).

Cell Death Analysis

Patterns of DNA damage were analyzed following drug treatment.

DNA Fragmentation Assay. Prostate cancer cell lines were exposed to 2.5 and 5 mM PB or PA for 2, 4, and 5 days. All cells (adherent and floating) were harvested, and the sample was divided equally. Half of the cells were lysed with 10 mM Tris-1 mM EDTA (pH 7.4) and 0.2% Triton X-100 (Sigma), and the remaining cells were processed for flow cytometry (see below). Centrifugation, precipitation, and quantification of high and low molecular weight DNA were performed as described previously (19–22). The percentage DNA fragmentation was calculated using the following equation: %DNA fragmentation = DNA in supernatant/DNA in supernatant + DNA in pellet or low molecular weight DNA/total DNA extracted.

Direct Fluorescence Detection of Digoxigenin-labeled Genomic DNA by Flow Cytometry. Using the ApopTag S7110 in situ apoptosis detection kit and protocol (Oncor, Inc., Gaithersburg, MD), samples were prepared for flow cytometry. Using flow cytometry (FACStar Plus; Becton Dickinson, San...
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Jose, CA), populations of apoptotic and nonapoptotic nuclei were quantified. The ratio of apoptotic nuclei to all nuclei counted determined the apoptotic index. Dexamethasone (1 μM)-treated human peripheral blood lymphocytes and terminal deoxynucleotidyltransferase enzyme-excluded samples acted as positive and negative controls, respectively (20).

DNA Laddering by Gel Electrophoresis. Prostate cancer cell lines were exposed to 2.5 mM PB for 2, 4, and 5 days. Cells were harvested after removal of media and nonadherent cells, lysed with 10 mM Tris (pH 8), 5 mM EDTA, and 0.5% Triton X-100 (Sigma), centrifuged, and incubated with 20 μg/ml DNase-free RNase (Boehringer Mannheim, Indianapolis, IN; 500 μg/ml). The supernatant was then treated with 10% SDS and 300 μg proteinase K/ml (Boehringer Mannheim; 16.4 mg/ml) for 1 h at 50°C. DNA was precipitated, washed, and resuspended. DNA was stained with ethidium bromide after 2% agarose gel electrophoresis.

Evaluation of Tumor Growth in Rats Exposed to PB in Their Drinking Water

Four groups of 10 male Copenhagen rats (Harlan-Sprague-Dawley, Indianapolis, IN), aged 12–16 weeks, were inoculated with 1 × 10⁴ Dunning R-3327 MAT-LyLu cells in the right hind leg. Three days later, PB in final concentrations of 2, 50, and 100 mM was added to the drinking water. Rats in the control arm received sodium acetate at 50 mM in their drinking water. On average, each rat consumed 6–8 ml water/day. Rats were evaluated twice weekly for tumor development and progression by following the size of the tumor at the inoculum site. Investigators measuring tumor size by calipers were blinded to treatment arm and PB dose.

![Fig. 3](https://example.com/f3.png)  
Dose-related phenotypic changes in PC-3 prostate cancer cells after PB treatment, a, untreated PC-3 cells; b, 2.5 mM PB-treated PC-3 cells; and c, 5 mM PB-treated PC-3 cells for 72 h, fixed and stained with hematoxylin and eosin (× 160).

Table 1  Percentage of DNA fragmentation of PB-treated TSU prostate cancer cells

<table>
<thead>
<tr>
<th>TSU prostate cancer cell line</th>
<th>Day</th>
<th>Control</th>
<th>2.5 mM PB</th>
<th>5 mM PB</th>
<th>2.5 mM PA</th>
<th>5 mM PA</th>
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<td>2</td>
<td>3.28</td>
<td>9.46</td>
<td>18.01</td>
<td>4.36</td>
<td>5.47</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3.68</td>
<td>18.11</td>
<td>22.24</td>
<td>3.72</td>
<td>4.03</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>3.23</td>
<td>26.38</td>
<td>26.12</td>
<td>4.78</td>
<td>5.67</td>
<td></td>
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</table>
RESULTS

Comparison of PB with PA and BU for Cytotoxic Effects. Growth inhibition assays were conducted using the Dunning rat R-3327 MAT-LyLu subline and the human prostate cancer cell lines TSU, PC-3, PPC-1, LNCaP-ATCC, and LNCaP-GW. Twofold dilutions from 20 to 0.185 mM were tested for each drug.

Fig. 1 shows the composite data for each cell line, drug, and concentration after a 4-day exposure. The IC_{50}s are 2.5 mM PB in the TSU and LNCaP cell lines and 5 mM PB in the Mat-LyLu and PC-3 cell lines. The IC_{50} for BU is similar to that for PB and is 5 mM or less in the MAT-LyLu, TSU, and LNCaP-GW cell lines. This contrasts with PA, for which the IC_{50} is 10 mM for Mat-LyLu and PC-3 cell lines, with the other cell lines requiring a far greater concentration than that which is tolerated clinically (10–20 mM). At 10 mM PB, all six of the cell lines tested have <50% growth inhibition. At clinically achievable levels, PB possesses greater potency against all tested prostate cancer cell lines compared with PA. BU is relatively equipotent to PB in every case in inducing growth inhibition or cell death.

The prostate cancer cell lines tested have heterogeneous p53, ras, and Rb mutations (23, 24), which may affect cellular and molecular responses to drug exposure. For example, the LNCaP-ATCC and LNCaP-GW lines differ; LNCaP-GW cells possess a p53 mutation (codon 273, arg → his; Ref. 23). Despite this, LNCaP-ATCC and LNCaP-GW cells are equally sensitive to the inhibitory effects of PB at 2.5 and 5 mM (Fig. 1). Overall, p53 status does not seem to play a major role in the activity or effect of these drugs, given the minimal difference in effect between PB and BU and no difference in terms of PA effect in the LNCaP variants.

An adriamycin-resistant TSU cell line (TSU-Adr 1000) was used to determine whether P-glycoprotein (MDR1) overexpression confers PB resistance. Using the growth inhibitory assay, wild-type TSU and TSU-Adr 1000 cell lines were exposed to equivalent concentrations of PB, vinblastine, or etoposide. Of note, both vinblastine and etoposide are used clinically in prostate cancer. Although the TSU-Adr 1000 cell line was resistant to vinblastine and etoposide, it was growth inhibited by PB (IC_{50}, 2.5 mM). The MDR1 status of this cell line did not affect its response to PB, suggesting that PB may be useful in cancers with high levels of MDR1 expression.

Phenotypic Changes after PB, PA, and BU Treatment in Vitro. Hematoxylin and eosin stains demonstrated clear, dose-related, phenotypic changes for each agent and similar changes for PB and BU at the same doses. Phenotypic changes in the TSU cell line exposed to media or 2.5 mM PB, BU, and PA are shown in Fig. 2. Cell numbers reflect the growth inhibitory effects of each agent with PB and BU, demonstrating dramatic inhibition (Fig. 1B). The treated cells demonstrate cellular flattening and extension of pseudopodia. The cytoplasm becomes vacuolized. In work by Samid et al. (3), these vacuoles are shown to contain lipid droplets after exposure to PA at a dose of 5 mM. In this study, the nuclei of PB-treated prostate cancer cells flatten as well, and nucleoli are well preserved. These histological changes in prostate cancer are consistent with cellular effects accompanying prostate cell apoptosis, as reported by Isaacs (25).

Fig. 3 demonstrates the dramatic phenotypic changes of the PC-3 line exposed to increasing concentrations of PB for 72 h. A marked increase in cells showing cytoplasmic extension, cytoplasm vacuolization, and nuclear disruption with multinucleated cells is apparent. These changes correlate with the growth inhibition assay for PC-3 shown in Fig. 1C. Of note, at 2.5 mM PB, the IC_{50} has not been reached, yet the distinct changes related to drug exposure and associated with cellular injury are seen clearly. Similar data showing phenotypic
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Inhibition of DNA Synthesis. After treatment with PB, the TSU cell line demonstrates marked reduction in DNA synthesis by \(^{[3]}H\)thymidine incorporation over the first 24 h and remains depressed, compared with controls, for as long as 96 h (data not shown). Samid et al. (3) has shown previously that DNA synthesis is inhibited after treatment with PA.

PB Induces Apoptosis. Low molecular weight DNA fragments are a marker of endonuclease activity accompanying apoptosis. PB induces apoptosis in the five prostate cancer cell lines tested (PC-3, TSU, LNCaP, DU-145, and PPC-1) at 2.5 mM (2–6-fold increase in the percentage of fragmented DNA over control at each time point) and at 5 mM (2.5–10 fold increase). PA induces DNA fragmentation above controls infrequently, and in only two cell lines (LNCaP and PPC-1) does it increase DNA fragments 1.3–2 times control. Table 1 demonstrates the raw data for the TSU prostate cancer line.

The PB-induced DNA fragmentation data are confirmed by flow cytometric analysis of digoxigenin-labeled, genomic DNA. Fig. 4 demonstrates both fragmented low molecular weight DNA and antidigoxigenin-positive cells as measures of apoptosis in the TSU cell line after exposure to PA and PB. The percentage of apoptotic nuclei after PB exposure increases 2–10 fold over controls, whereas PA does not induce apoptosis over controls as demonstrated by antidigoxigenin antibody fluorescence. Fig. 5 demonstrates the correlation between the DNA fragmentation assay and the antidigoxigenin antibody staining \((r = 0.688)\) and segregates PB from PA as an inducer of prostate cancer apoptosis clearly.

PB Induced Apoptosis in Prostate Cancer Cell Lines. The time course of the effect of PB on prostate cancer cells begins within the first 24 h of exposure, with a reduction in DNA synthesis. The morphological changes of cytoplasmic vacuolization and nuclear swelling occur after 48–72 h of continuous PB treatment. Finally, PB induces cell death via apoptosis as early as 48 h, continuing for at least 120 h of drug exposure.

In Vivo Evaluation of PB as a Cytotoxic Agent in Prostate Cancer. After identifying the time course of the effect of PB on human prostate cancer in vitro, we tested PB in vivo. The Dunning rat R-3327 MAT-LyLu prostate cancer line, which doubles every 24–36 h (26) and is androgen independent, was used. Fig. 7 shows a dose-related inhibition of tumor growth in animals with PB-supplemented drinking water. Rats with 100 mM PB-supplemented drinking water had a 30–45% reduction in tumor size compared with controls. This difference was statistically significant \((P < 0.026\) by paired \(t\) test). PB at 100

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Changes at doses lower than the IC\(_{50}\) were observed for the other cell lines: PPC-1, LNCaP-ATCC, and LNCaP-GW.

Fig. 5. Comparison of the ability of PB and PA to induce apoptosis. The plot compares the fold increase of low molecular weight DNA fragmentation over the fold increase in apoptotic nuclei of each prostate cancer cell line after treatment with PA or PB. PB-treated prostate cancer cell lines undergo apoptosis, as demonstrated by these techniques. PA does not induce significant apoptosis.

Fig. 6. Agarose gel electrophoretic patterns of DNA isolated from PB-treated and untreated TSU prostate cancer cells. Lane A, 123-bp marker; Lane B, λ-HindIII marker; Lane C, blank; Lane D, untreated TSU cells harvested at 96 h; Lane E, 2.5 mM PB-treated TSU cells for 48 h; Lane F, 2.5 mM PB-treated TSU cells for 96 h; and Lane G, 2.5 mM PB-treated TSU cells for 120 h.
PB is an active differentiating agent and cytotoxic compound available for clinical testing in human prostate cancer therapy. In this report, we demonstrate for the first time that PB has cytotoxic effects and can induce apoptosis in human prostate cancer cell lines. Therefore, PB is an attractive candidate to develop as a novel, lead compound for the therapy of prostate cancer.

We found that PB is more potent at inhibiting the growth of the human prostate cancer cell lines than PA. PB inhibits prostate cancer growth at clinically achievable levels, whereas the concentration of PA required to inhibit cell growth in vitro is at the upper limits of the plasma concentrations produced by tolerated doses of the agent in patients. Morphological changes suggesting injury due to PB can be seen at doses below the IC_{50}. Also, PB induces its effect on prostate cancer cell lines independent of p53 mutational status or MDR1 overexpression. PB increases the apoptotic rate in prostate cancer cell lines over controls dramatically, 2–10 times the basal rate of apoptosis. Even a slight modification in apoptotic death rates may translate to the clinical significance of PB as an antitumor agent (27, 28). PB as monotherapy can also slow the growth of transplanted prostate tumors in rats when given in the rats’ drinking water. PA and PB have been reported to reduce anaerobic glycolysis, increase cyclic AMP, induce apoptosis, alter growth factor and hormone expression, increase histone acetylation, and alter chromatin conformation (17, 32–34). Nuclear swelling is quite dramatic and consistent in all the cell lines after exposure to either PB or BU. Like BU, PB can induce apoptosis, as demonstrated by fragmentation of genomic DNA. These effects associated with both BU and PB are primarily cytotoxic and may be related to the induction of a differentiated state. Our studies show that a pathway of PB drug activity includes signaling cancer cell apoptosis. This finding is consistent with the studies of Heerdt et al. (35), who have described the role of short chain fatty acids and BU in the differentiation and subsequent induction of apoptosis in human colon carcinoma cell lines.

PB is β oxidized in vivo to PA in the mitochondria of cells rapidly (12). Pharmacokinetic data from bolus human studies of PB suggest that PA and PB may have distinct pharmacological and pharmacokinetic properties (14). Thus, PB may induce an effect at the molecular level before it is metabolized to PA. Evaluation of bioactivity of PB in clinical trials for pure PB effects remains a challenge given its metabolism to PA. Determination of distinct in vitro PB versus PA effects on the expression of PSA, interleukin-6, endothelin-1, and other markers of prostate cancer morbidity may aid in identifying bioactivity unique to PB in vivo (36, 37).

PB is currently in Phase I testing for patients with refractory solid tumors. Phase I testing will determine toxicities and will evaluate pharmacokinetics of both i.v. and oral formulations. Theoretically, differentiating agent therapies or even antimetastatic therapies require continuous exposure of the agent to maintain the desired effect of manipulating established tumor sites. Preventive therapies are likely to require continuous ex-
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